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Evidence for Autotrophic CO_2 Fixation via the Reductive Tricarboxylic Acid Cycle by Members of the ε Subdivision of Proteobacteria†

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Based on 16S rRNA gene surveys, bacteria of the ε subdivision of proteobacteria have been identified to be important members of microbial communities in a variety of environments, and quite a few have been demonstrated to grow autotrophically. However, no information exists on what pathway of autotrophic carbon fixation these bacteria might use. In this study, *Thiomicrospira denitrificans* and *Candidatus* Arcobacter sulfidicus, two chemolithoautotrophic sulfur oxidizers of the ε subdivision of proteobacteria, were examined for activities of the key enzymes of the known autotrophic CO_2 fixation pathways. Both organisms contained activities of the key enzymes of the reductive tricarboxylic acid cycle, ATP citrate lyase, 2-oxoglutarate:ferredoxin oxidoreductase, and pyruvate:ferredoxin oxidoreductase. Furthermore, no activities of key enzymes of other CO_2 fixation pathways, such as the Calvin cycle, the reductive acetyl coenzyme A pathway, and the 3-hydroxypropionate cycle, could be detected. In addition to the key enzymes, the activities of the other enzymes involved in the reductive tricarboxylic acid cycle could be measured. Sections of the genes encoding the α- and β-subunits of ATP citrate lyase could be amplified from both organisms. These findings represent the first direct evidence for the operation of the reductive tricarboxylic acid cycle for autotrophic CO_2 fixation in ε-proteobacteria. Since ε-proteobacteria closely related to these two organisms are important in many habitats, such as hydrothermal vents, oxic-sulfidic interfaces, or oilfields, these results suggest that autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle might be more important than previously considered.

Almost all major groups of prokaryotes include representatives that are able to grow autotrophically (33). These organisms play an essential role in ecosystems by providing a continuous supply of organic carbon for heterotrophs. The Calvin-Benson-Bassham cycle (Calvin cycle) represents the most important extant autotrophic carbon fixation pathway (43, 50). Despite its global significance, it is restricted to organisms with high-energy yield from a chemotrophic or phototrophic lifestyle. Microorganisms present in extreme environments, e.g., high temperature or anaerobic or acidic conditions, generally utilize different CO_2 fixation pathways (17, 33). At present, there are three alternative pathways known: the reductive tricarboxylic acid (TCA) cycle, the reductive acetyl coenzyme A (CoA) pathway, and the 3-hydroxypropionate cycle (4, 33).

It has been proposed that the first autotrophic pathway was akin to either the reductive TCA cycle or the reductive acetyl-CoA pathway (11, 17, 35, 45, 58). The reductive TCA cycle has the characteristics of an autocatalytic cycle and leads to a complex cyclic reaction network from which other anabolic pathways could have evolved (11, 58): e.g., the oxidative TCA cycle (8, 45). Based upon biochemical and isotopic analyses, the reductive TCA cycle appears to operate in phylogenetically diverse autotrophic bacteria and archaea, including genera of anoxic phototrophic bacteria (*Chlorobium*) (14, 18, 28), sulfate-reducing bacteria (*Desulfobacter*) (48), microaerophilic, hyperthermophilic hydrogen-oxidizing bacteria (*Aquifex* and *Hydrogenobacter*) (5, 49), and sulfur-reducing *Crenarchaeota* (*Thermoproteus* and *Pyrobaculum*) (5, 24, 46). The reductive TCA cycle is essentially the oxidative TCA cycle running in reverse, leading to the fixation of two molecules of CO_2 and the production of one molecule of acetyl-CoA (Fig. 1). Acetyl-CoA is reductively carboxylated to pyruvate, from which all other central metabolites can be formed. Most of the enzymes of the two pathways are shared, with the exception of three key enzymes that allow the cycle to run in reverse: ATP citrate lyase, 2-oxoglutarate:ferredoxin oxidoreductase, and fumarate reductase. 2-Oxoglutarate:ferredoxin oxidoreductase catalyzes the carboxylation of succinyl-CoA to 2-oxoglutarate, ATP citrate lyase the ATP-dependent cleavage of citrate to acetyl-CoA and oxaloacetate, and fumarate reductase the reduction of fumarate forming succinate. The presence of these enzyme activities in autotrophically grown bacteria and archaea is indicative of a functioning reductive TCA cycle (5, 28, 46, 48, 49). However, activity of ATP citrate lyase has also been identified in heterotrophic and facultative autotrophic organisms: i.e., the sulfate reducers *Desulfobacter postgatei* and *Desulfobacter hydrogenophilus*, respectively (37, 48). In *D. postgatei* and *D. hydrogenophilus* growing on acetate, ATP citrate lyase catalyzes citrate synthesis, so ATP can be formed via substrate-level phosphorylation, increasing the energy yield of the bacterium. ATP citrate lyase also exists in eukaryotes, where it plays a role in supplying cytosolic acetyl-CoA for biosynthesis of fatty acids and cholesterols (15).

The only cultured bacteria or archaea for which a complete

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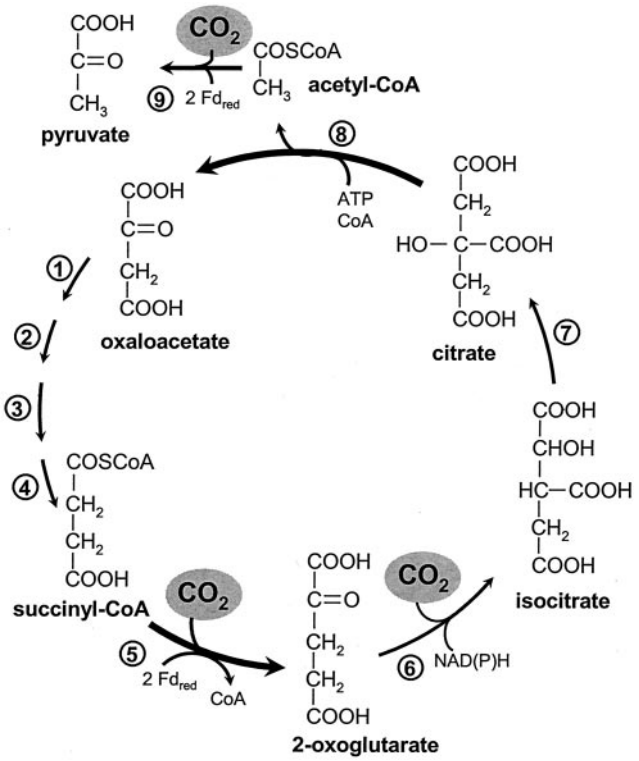

FIG. 1. Outline of the reductive citric acid cycle for autotrophic CO_2 fixation. The reactions catalyzed by key enzymes are indicated by bold arrows. Enzyme activities: 1, malate dehydrogenase (EC 1.1.1.37); 2, fumarate hydratase (fumarase) (EC 4.2.1.2); 3, fumarate reductase; 4, succinyl-CoA synthetase (EC 6.2.1.5); 5, 2-oxoglutarate:ferredoxin oxidoreductase (EC 1.2.7.3); 6, isocitrate dehydrogenase (EC 1.1.1.42); 7, aconitate hydratase (aconitase) (EC 4.2.1.3); 8, ATP citrate lyase (EC 2.3.3.8); and 9, pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1). Fd_{red}, reduced ferredoxin.

gene encoding ATP citrate lyase has been identified to date are the green-sulfur bacteria *Chlorobium tepidum* and *Chlorobium limicola* (13, 29). The ATP citrate lyase of *Chlorobium* is an $\alpha_4\beta_4$-heteromeric enzyme encoded by two separate genes, *aclA* (large α-subunit; 1,827 bp) and *aclB* (small β-subunit; 1,197 bp), which are adjacent to one another in the genome (13, 29). In *Hydrogenobacter thermophilus*, the ATP-dependent cleavage of citrate is catalyzed by the combined action of two recently identified enzymes, citryl-CoA synthetase (homolog to succinyl-CoA synthetase) and citryl-CoA lyase (homolog to citrate synthase) (2, 3), possibly representing the ancient state of ATP-dependent citrate cleavage enzyme.

In recent years, the ε subdivision of the proteobacteria has received increased attention as 16S rRNA genes phylogenetically associated with ε-proteobacteria have been frequently obtained from a variety of environments, including hydrothermal vents, the oxic-sulfidic interfaces of anoxic marine basins (Cariaco Basin and Black Sea), and oilfields, indicating their importance in these systems (22, 23, 32, 34, 38, 44, 55, 57). Based on the physicochemical environment, it has been speculated that these ε-proteobacteria could potentially be autotrophs, which is further supported by the recent isolation of several chemolithoautotrophic ε-proteobacteria (1, 9, 26, 30, 36, 53, 54, 60). However, no information exists what pathway of autotrophic carbon fixation these bacteria might use. Recently, genes coding for ATP citrate lyase have been identified on two fosmids also containing the 16S rRNA of the main ε-proteobacterial epibionts of *Alvinella pompejana*—a polychaete living on sulfide structures at deep-sea hydrothermal vents on the East Pacific Rise—suggesting that these bacteria use the reductive TCA cycle either for autotrophic CO_2 fixation or for assimilation of organic carbon (10). On these fosmids, the genes corresponding to *aclA* and *aclB* were adjacent to each other in an arrangement identical to that of *Chlorobium*.

In this study, we investigated the CO_2 fixation pathway of *Thiomicrospira denitrificans* and of *Candidatus* Arcobacter sulfidicus, two autotrophic sulfur-oxidizing bacteria belonging to the ε subdivision of proteobacteria (39, 60). We demonstrated the activities of the enzymes of the reductive TCA cycle in both organisms. Key enzyme activities of other autotrophic pathways could not be detected. This provides evidence that these bacteria use the reductive TCA cycle for autotrophic CO_2 fixation. Considering the predominance of ε-proteobacteria in a variety of habitats, this suggests that the autotrophic fixation of CO_2 through the reductive TCA cycle might be more widespread and thus more significant than previously thought.

MATERIALS AND METHODS

Bacteria and growth conditions. *T. denitrificans* (DSM 1251) was grown autotrophically in 2-liter glass bottles at 25°C under denitrifying conditions according to Timmer-Ten Hoor (56). Thiosulfate served as an electron donor, nitrate as an electron acceptor, and sodium bicarbonate as the sole carbon source. The average doubling time was 20 h. *Candidatus* Arcobacter sulfidicus can at present only be cultivated in a special growth apparatus, resulting in the fact that no transferable pure cultures of *Candidatus* Arcobacter sulfidicus are maintained on a routine basis (60). For the present investigation, we initiated new enrichments from purified cell suspensions that were harvested previously from a purified cell culture (60) and stored in our laboratory. The purified cells were grown autotrophically in a flowthrough system at 25°C under microaerophilic conditions using H_2S as an electron donor, O_2 as an electron acceptor, and CO_2 as a carbon source (60). Since the organism is growing in an open system, it is difficult to determine the actual growth rate, but CO_2 fixation experiments demonstrated a fixation rate of 1.04×10^{-6} nmol of C $cell^{-1}$ h^{-1} (60). *Chloroflexus aurantiacus* OK-70-fl (DSM 636) (51), *Desulfobacterium autotrophicum* (DSM 3382) (31), *Desulfobacter hydrogenophilus* (DSM 3380) (59), and *Ralstonia eutropha* JPM 134 (DSM 4058) (16) were grown as described in the references. The biomass of anaerobic and microaerophilic organisms was harvested under the exclusion of oxygen. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, Calif.) was grown on Luria-Bertani medium. Kanamycin was added to a final concentration of 50 μg/ml.

Syntheses. Malonyl-CoA and 3-hydroxypropionate were synthesized as described elsewhere (21) and were stored at −20°C.

Preparation of cell extracts. Cell extracts were prepared using a mixer mill (type MM2; Retsch, Haare, Germany). Four ml buffer (100 mM Tris HCl, pH 7.8, 3 mM 1,4-dithioerythritol [DTE], 250 μl protease inhibitor cocktail for bacterial cells [Sigma-Aldrich, Saint Louis], and 1 mg of DNase I per 10 ml of buffer) was added per 1 g of wet cells. Aliquots (0.6 ml) of the suspension were placed in 1.5-ml plastic microcentrifuge vials; for anaerobic preparations, 1-ml stoppered glass vials were used and 1 g of glass beads (diameter, 0.1 to 0.25 mm) was added to the solution. From *Candidatus* Arcobacter sulfidicus, only a small amount of cells was available. In this case, 20 mg of cells was resuspended in 100 μl of buffer (see above). The suspension was placed in a 0.5-ml microcentrifuge vial, and 100 mg glass beads was added. The ice-cold solution was treated in the mixer mill for 7 min at 100% intensity (30 Hz). Following a centrifugation step (10 min, 12,000 × *g*, 4°C), the supernatant was used for enzyme tests. Protein concentration in cell extracts was determined by the method of Bradford (6) using bovine serum albumin as a standard.

Enzyme assays. Routinely, enzyme assays (0.5-ml assay mixture) were performed in stoppered 0.5-ml glass cuvettes or 1-ml glass vials. The assay temperature was 25°C. Reactions involving pyridine nucleotides were followed spectrophotometrically at 365 nm: $\varepsilon_{365\ nm}(NAD(P)H) = 3.4 \times 10^3\ M^{-1}\ cm^{-1}$.

Reactions involving methyl viologen (MV) or benzyl viologen (BV) were followed spectrophotometrically at 578 nm: ε_{578} (MV) = 9.8×10^3 M^{-1} cm^{-1}, and ε_{578} (BV) = 8.6×10^3 M^{-1} cm^{-1} (12).

(i) ATP citrate lyase (reductive TCA cycle). ATP citrate lyase activity was determined by coupling the reaction to malate dehydrogenase activity, which reduces the produced oxaloacetate with NADH. The citrate-, CoA-, and MgATP-dependent oxidation of NADH was monitored. The assay mixture contained 100 mM MOPS (morpholinepropanesulfonic acid)/NaOH, pH 8.3, 5 mM DTE, 5 mM $MgCl_2$, 3 mM ATP, 0.5 mM CoA, 0.4 mM NADH, 1 U malate dehydrogenase (Sigma-Aldrich), and 3 mM D-citrate. The reaction was started by the addition of citrate.

(ii) 2-Oxoglutarate:acceptor oxidoreductase and pyruvate:acceptor oxidoreductase (reductive TCA cycle). The 2-oxoglutarate- or pyruvate- and CoA-dependent reduction of oxidized BV was monitored. The following anaerobic assay mixture was used with N_2 gas as headspace: 100 mM Tris-HCl, pH 7.8, 4 mM DTE, 2 mM $MgCl_2$, 0.5 mM CoA, 2 mM BV, and 3 mM 2-oxoglutarate or pyruvate. Dithionite was added by syringe from 5 mM stock solution until a faint bluish color was obtained. The reaction was started by the addition of 2-oxoglutarate or pyruvate. Two viologen dyes were reduced per 2-oxoacid transformed.

(iii) Fumarate reductase (reductive TCA cycle). The anaerobic assay mixture contained 100 mM Tris-HCl, pH 7.8, 4 mM DTE, 2 mM $MgCl_2$, 2 mM reduced BV, and 1 mM fumarate. Reduced viologen dye was obtained by adding dithionite (50 mM solution) until an absorption of 1.5 at 578 nm was obtained. The reaction was started by the addition of fumarate. Two viologen dyes were oxidized per fumarate transformed.

(iv) Malate dehydrogenase (reductive and oxidative TCA cycle). The oxaloacetate-dependent oxidation of NADPH or NADH was monitored (24).

(v) Isocitrate dehydrogenase (reductive and oxidative TCA cycle). The isocitrate-dependent reduction of $NADP^+$ was monitored (24).

(vi) Fumarate hydratase (reductive and oxidative TCA cycle). Fumarate consumption was monitored spectrophotometrically at 240 nm (ε_{240} = 2,440 M^{-1} cm^{-1}) (24).

(vii) 2-Oxoglutarate dehydrogenase and pyruvate dehydrogenase (oxidative TCA cycle). In the oxidative TCA cycle, 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase replace 2-oxoglutarate:acceptor oxidoreductase and pyruvate:acceptor oxidoreductase. The 2-oxoglutarate- or pyruvate- and CoA-dependent reduction of NAD^+ or $NADP^+$ was monitored. The test mixture contained 100 mM Tris-HCl, pH 7.8, 5 mM DTE, 5 mM $MgCl_2$, 1 mM NAD^+ or $NADP^+$, 0.5 mM CoA, 0.5 mM thiamine pyrophosphate, and 5 mM 2-oxoglutarate or pyruvate. The addition of 2-oxoglutarate or pyruvate started the reaction.

(viii) Malonyl-CoA reduction to 3-hydroxypropionate (3-hydroxypropionate cycle). The malonyl-CoA-dependent oxidation of NADPH was monitored (24).

(ix) Reduction of 3-hydroxypropionate to propionyl-CoA (3-hydroxypropionate cycle). The 3-hydroxypropionate-, CoA-, and MgATP-dependent oxidation of NADPH was followed (24).

(x) Carbon monoxide dehydrogenase (reductive acetyl-CoA pathway). The anaerobic assay mixture contained 100 mM Tris-HCl, pH 7.8, 4 mM DTE, 2 mM $MgCl_2$, 4 mM MV, and CO gas (1-bar headspace). Dithionite was added by syringe from 5 mM stock solution until a faint bluish color was obtained. The reaction was started by the addition of protein. In control experiments, CO was replaced by N_2 gas.

(xi) RubisCO. The carboxylation of ribulose 1,5-bisphosphate with $^{14}CO_2$ was followed by measuring the substrate-dependent incorporation of ^{14}C from [^{14}C]bicarbonate into acid-stable products (24). *Ralstonia eutropha*, a facultative autotroph expressing ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity also while growing on fructose as an organic C source, served as a positive control (5).

DNA extraction, PCR amplification, cloning, and sequencing of ATP citrate lyase. Chromosomal DNA was extracted with the "SoilMaster DNA extraction kit" (Epicentre, Madison, WI) according to the provided protocol. Degenerate primers were designed based on an alignment of known ATP citrate lyase genes. An ~1,000-bp fragment of the gene for the ATP citrate lyase α-subunit (*aclA*) was directly amplified from the purified DNA of *Candidatus* Arcobacter sulfidicus and *Thiomicrospira denitrificans* in a 35-cycle PCR at an annealing temperature of 42°C with degenerate primers (F2, 5′-TGCATAGCAATHGGNGGN GA-3′; and R5, 5′-CCGATAGANCCRTCNACRTT-3′). A second ~340-bp fragment of the gene for the ATP citrate lyase β-subunit (*aclB*) was amplified from both cultures using primers 892F and 1204R and conditions according to Campbell et al. (10). PCR products were purified from agarose gels using a QIAquick gel extraction kit (QIAGEN, Inc., Chatsworth, CA) and cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) as described by the manufacturer. Colonies were picked, and the plasmid DNA was purified with the QIAprep spin miniprep kit (QIAGEN) as described by the manufacturer. Plasmids were sent to the BioResource Center at Cornell University (Ithaca, NY) for sequencing. DNA sequencing was performed using the Applied Biosystems Automated 3730 DNA analyzer. The big dye terminator chemistry and AmpliTaq-FS DNA polymerase were used.

Phylogenetic analysis. Sequences were loaded into MacVector (Accelrys Inc., San Diego, CA), and aligned using the ClustalW program implemented in MacVector. Aligned sequences were imported into PAUP, version 4.0b10, for phylogenetic analysis (52). Models for use in minimum-evolution and maximum-likelihood searches were chosen using the likelihood ratio test in the Modeltest program, version 3.5 (42). The chosen substitution model corresponded to the GTR + G model: six classes of substitution and unequal base frequencies (GTR; general time reversible), the proportion of invariant sites set at 0, and the evolutionary rate of sites varying according to a gamma distribution (G). The specific model parameters are available upon request. Trees were inferred using distance analysis with minimum evolution as the optimal criterion and the distance measure set to maximum likelihood, parsimony analysis, and maximum-likelihood analysis. Heuristic searches under the minimum-evolution and maximum-parsimony criterion were performed using 1,000 random-addition replicates with tree bisection-reconnection branch swapping. Bootstrapping of minimum-evolution and maximum-parsimony analyses used 1,000 bootstrap replicates of 10 random addition replicates each. Heuristic searches under the maximum-likelihood criterion were performed using five random addition replicates with tree bisection-reconnection branch swapping.

Nucleotide sequence accession number. The nucleotide sequences have been deposited in GenBank. The accession numbers of the section of *aclA* coding for the large ATP citrate lyase α-subunit and *aclB* coding for the small ATP citrate lyase β-subunit of *T. denitrificans* are AY885676 and AY885677, respectively. The accession number of the section coding for the ATP citrate lyase α-subunit of *Candidatus* Arcobacter sulfidicus is AY885678.

RESULTS

Enzyme measurements in *T. denitrificans* and *Candidatus* Arcobacter sulfidicus. Cell extracts of *T. denitrificans* and *Candidatus* Arcobacter sulfidicus were tested for key enzyme activities of the known autotrophic CO_2 fixation pathways. These included ribulose 1,5-bisphosphate carboxylase/oxygenase, the key enzyme of the Calvin cycle, and CO dehydrogenase, the main enzyme of the reductive acetyl-CoA pathway. To test the occurrence of the reductive TCA cycle, ATP citrate lyase, 2-oxoglutarate:ferredoxin oxidoreductase, and pyruvate:ferredoxin oxidoreductase were measured. The latter enzyme is also present in organisms using the reductive acetyl-CoA pathway for autotrophic CO_2 fixation. For the 3-hydroxypropionate cycle, the NADPH-dependent reduction of malonyl-CoA to 3-hydroxypropionate (malonyl-CoA reductase) and the reductive transformation of 3-hydroxypropionate (propionyl-CoA synthase) were tested.

In both organisms, no ribulose 1,5-bisphosphate carboxylase/oxygenase activity was detected. This confirms earlier results for *Candidatus* Arcobacter sulfidicus (60). The assay conditions were controlled with extracts of *Ralstonia eutropha* which exhibited a specific activity of around 100 nmol min^{-1} mg cell $protein^{-1}$, when measured at 25°C. Also no CO dehydrogenase activity was detected in both bacteria. This enzyme of the reductive acetyl-CoA pathway was controlled by using extract of *D. autotrophicum* which had an activity of 900 nmol min^{-1} mg cell $protein^{-1}$ at 25°C. The key enzymes of the 3-hydroxypropionate cycle could not be detected either. In this case, a control at 25°C was not possible since only *C. aurantiacus* and members of the *Sulfolobaceae* are known to use this pathway and they grow at higher temperatures (24, 25, 27, 51). Extract of *C. aurantiacus* was used as a control, but the enzyme activities of malonyl-CoA reductase (160 nmol min^{-1} mg cell

TABLE 1. Specific activities (nmol min^{-1} mg cell $protein^{-1}$) of key enzymes of autotrophic CO_2 fixation pathways[a]

Enzyme activity tested	Sp act (nmol min^{-1} mg $protein^{-1}$)		
	Thiomicrospira denitrificans	*Candidatus* Arcobacter sulfidicus	Control
Calvin cycle			
RubisCO	<1	<1	100 (*R. eutropha*)
Reductive acetyl-CoA pathway			
CO dehydrogenase (MV)	<2	<1	900 (*D. autotrophicum*)
Pyruvate:acceptor (BV) oxidoreductase	340	170	560 (*D. autotrophicum*)
3-Hydroxypropionate cycle			
Malonyl-CoA reductase	<1	<1	160 (*C. aurantiacus*)
Propionyl-CoA synthase	<1	<1	100 (*C. aurantiacus*)
Reductive citric acid cycle			
ATP citrate lyase	65	45	900 (*D. hydrogenophilus*)
2-Oxoglutarate:acceptor (BV) oxidoreductase	360	175	510 (*D. hydrogenophilus*)
Pyruvate:acceptor (BV) oxidoreductase	340	170	330 (*D. hydrogenophilus*)

[a] Mean values were obtained from three independent experiments. Standard errors were less than ±20%. The assays were carried out at 25°C.

$protein^{-1}$) and propionyl-CoA synthase (100 nmol min^{-1} mg cell $protein^{-1}$) were measured at 55°C.

In contrast, key enzymes of the reductive TCA cycle were present in cell extracts of both organisms (Table 1). ATP citrate lyase activity was detected with a specific activity of 65 nmol min^{-1} mg cell $protein^{-1}$ in *T. denitrificans* and 45 nmol min^{-1} mg cell $protein^{-1}$ in *Candidatus* Arcobacter sulfidicus. For both organisms, the reaction was strictly dependent on ATP, CoA, and citrate. The ATP citrate lyase activity was measured in a spectrophotometrical assay coupled to malate dehydrogenase. To ensure that malate dehydrogenase was not limiting, exogenous enzyme was added to the reaction mixture. The highest activity of ATP citrate lyase in *T. denitrificans* was measured at pH 8.3, while at pH 7.8, only 50% activity remained. In addition to ATP citrate lyase, both 2-oxoglutarate and pyruvate oxidoreductase could be measured with benzyl viologen as an artificial electron acceptor. The specific activities of 2-oxoglutarate:acceptor (BV) oxidoreductase were 360 nmol min^{-1} mg cell $protein^{-1}$ for *T. denitrificans* and 175 nmol min^{-1} mg cell $protein^{-1}$ for *Candidatus* Arcobacter sulfidicus, respectively. The specific activities of pyruvate:acceptor (BV) oxidoreductase were similar, with values of 340 nmol min^{-1} mg cell $protein^{-1}$ in *T. denitrificans* and 170 nmol min^{-1} mg cell $protein^{-1}$ in *Candidatus* Arcobacter sulfidicus (Table 1). Both enzyme activities were strictly CoA and substrate dependent. In contrast to these enzyme activities, there were no indications for 2-oxoglutarate or pyruvate dehydrogenase activities, neither with NADP nor NAD as an electron acceptor.

In addition to these key enzymes, also other enzymes involved in the reductive TCA cycle were present in *T. denitrificans* (Table 2). Fumarate reductase was tested with reduced benzyl viologen as artificial electron donor, and its specific activity was 220 nmol min^{-1} mg cell $protein^{-1}$. Fumarate hydratase exhibited a specific activity of 390 nmol min^{-1} mg cell $protein^{-1}$ in the direct spectrophotometrical assay. Isocitrate dehydrogenase was NADP dependent, with specific activities of 1,100 nmol min^{-1} mg cell $protein^{-1}$ for *T. denitrificans* and 2,300 nmol min^{-1} mg cell $protein^{-1}$ for *Candidatus* Arcobacter sulfidicus, respectively. Both NADPH- and NADH-dependent malate dehydrogenase activities were found, with values of 830 and 960 nmol min^{-1} mg cell $protein^{-1}$ for *T. denitrificans* and 810 and 510 nmol min^{-1} mg cell $protein^{-1}$ for *Candidatus* Arcobacter sulfidicus, respectively.

Amplified genes. ATP citrate lyase is a key enzyme of the reductive TCA cycle in bacteria. We successfully amplified, cloned, and sequenced sections of the genes coding for the α-subunit (*aclA*) and the β-subunit (*aclB*) of ATP citrate lyase from both *Candidatus* Arcobacter sulfidicus and *T. denitrificans* by using a newly designed primer pair, F2/R5, for *aclA* and previously published primers for *aclB* (10). This resulted in

TABLE 2. Specific activities (nmol min^{-1} mg cell $protein^{-1}$) of enzymes of the reductive and oxidative TCA cycle[a]

Enzyme activity tested	Sp act (nmol min^{-1} mg $protein^{-1}$)	
	Thiomicrospira denitrificans	*Candidatus* Arcobacter sulfidicus
Fumarate reductase	220	n.d.
Fumarate hydratase	390	n.d.
Isocitrate dehydrogenase	1,100	2,300
Malate dehydrogenase		
NADPH	830	810
NADH	960	510
2-Oxoglutarate dehydrogenase		
$NADP^+$	<1	<1
NAD^+	<1	<1
Pyruvate dehydrogenase		
$NADP^+$	<1	<1
NAD^+	<1	<1

[a] Mean values were obtained from three independent experiments. Standard errors were less than ±20%. The assays were carried out at 25°C. n.d., not determined.

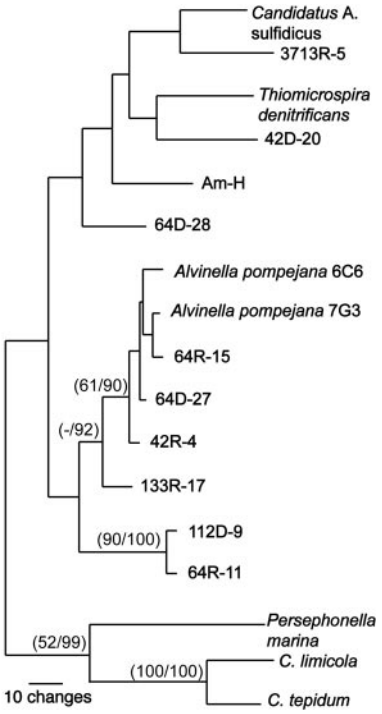

FIG. 2. Phylogenetic tree based on a 333-bp-long fragment (306 positions considered for the analyses) of the gene coding for the β-subunit of ATP citrate lyase (*aclB*). The tree depicts the phylogenetic relationship of *aclB* of *Candidatus* Arcobacter sulfidicus and *Thiomicrospira denitrificans* to other bacterial sequences derived directly either from the environment or other cultures. The tree was constructed by using distance analysis in PAUP*, version 4.0b10, with minimum evolution as the optimal criterion and the distance measure set to maximum likelihood. Trees constructed with other reconstruction algorithms (parsimony and maximum likelihood) resulted in the same overall topology. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replications. Bootstrap values from both distance analysis (first number) and parsimony analysis (second number) are depicted. Bootstrap values less than 50% are not shown. The scale bar represents 10 estimated nucleotide changes.

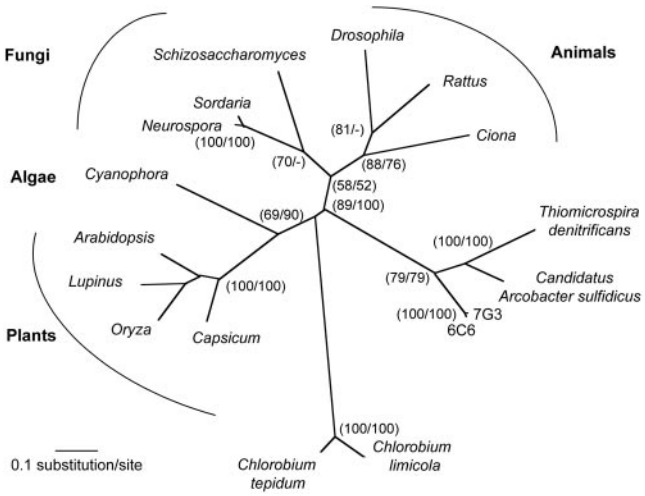

FIG. 3. Phylogenetic tree based on a 1,016-bp-long fragment (952 positions considered for the analyses) of the gene coding for the α-subunit of ATP citrate lyase (*aclA*). The tree depicts the phylogenetic relationship of *aclA* of *Candidatus* Arcobacter sulfidicus and *Thiomicrospira denitrificans* to other bacterial sequences (two *Chlorobium* species and two sequences [7G3 and 6C6] derived from the epibiont community of *Alvinella pompejana*), as well as eukaryotic sequences. The tree was constructed by using maximum-likelihood analysis in PAUP*, version 4.0b10. Trees constructed with other reconstruction algorithms (distance analyses and parsimony) resulted in the same overall topology. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replications. Bootstrap values from both distance analysis (first number) and parsimony analysis (second number) are depicted. Bootstrap values less than 50% are not shown. The positions of the sequences derived from the two *Chlorobium* species could not be confidently resolved with any treeing method due to their divergence from the other sequences. The scale bar represents 0.1 estimated change per nucleotide.

~1,000 bp of sequence for *aclA* (representing more than 50% of the expected ~1,800 bp) and ~330 bp of sequence for *aclB* (more than 25% of the expected ~1,200 bp). The *aclB* sequence of *T. denitrificans* is most closely related to the corresponding sequence of clone 42D-20 (90% sequence identity on protein level), whereas the *aclB* of *Candidatus* Arcobacter sulfidicus is most closely related to clone 3713R-5 (90% sequence identity on protein level), in general agreement with results of Campbell et al. (10) (Fig. 2). Both clone 42D-20 and clone 3713R-5 were amplified from the epibiont community of *Alvinella pompejana* (10). In congruence with 16S rRNA phylogeny, the *aclB* sequences from ε-proteobacteria fall in a monophyletic group separate from *Persephonella* (*Aquificales*) and *Chlorobium* (green sulfur bacteria). The primer pair F2/R5 amplified an ~1,000-bp-long fragment. Phylogenetic analyses of available *aclA* sequences show that *aclA* from *T. denitrificans* and *Candidatus* Arcobacter sulfidicus form a monophyletic group with the two sequences obtained from the epibiont community, separated from the *aclA* of eukaryotes (fungi, plants, and animals) and of *C. limicola* and *C. tepidum* (Fig. 3). The translated *aclA* sequences of *T. denitrificans* and *Candidatus* Arcobacter sulfidicus share 85% identity between each other. The identities between the translated *aclA* sequence from *T. denitrificans* and 7G3, *Neurospora crassa*, *Arabidopsis thaliana*, *Rattus norvegicus*, and *Chlorobium limicola* are 78.8%, 56.1%, 51.5%, 51.1%, and 35.9%, respectively (the respective values for the translated sequence of *Candidatus* Arcobacter sulfidicus are 80.9%, 57.4%, 54.3%, 50.8%, and 40.1%, respectively).

DISCUSSION

In this communication, we present evidence that *Candidatus* Arcobacter sulfidicus and *T. denitrificans*, two chemolithoautotrophic organisms that belong to the ε subdivision of the proteobacteria, use the reductive TCA cycle for autotrophic carbon fixation. All enzymes of the reductive TCA cycle could be measured in these organisms, including the key enzymes ATP citrate lyase and 2-oxoglutarate:ferredoxin oxidoreductase. Based on the doubling time of 20 h, a specific CO_2 fixation rate of 48 nmol min^{-1} mg cell $protein^{-1}$ follows for *T. denitrificans* (for calculation of specific CO_2 fixation rates of growing cultures see reference 21). Since the reductive TCA cycle including pyruvate synthesis contains three CO_2-fixing enzymes, the minimal specific activities of the enzymes of the pathway to sustain autotrophic growth should be 16 nmol

min^{-1} mg cell $protein^{-1}$. The measured specific activities of all enzymes of the cycle were higher than this minimal activity and can therefore account for the growth rate of autotrophically growing cells. Unfortunately, we cannot make the same argument for *Candidatus* Arcobacter sulfidicus, since the organism can presently only be consistently grown in a flowthrough system and thus we were not able to determine its actual growth rate. However, based on the measured CO_2 fixation rate of 1.04×10^{-6} nmol of C $cell^{-1}$ h^{-1} for an exponentially growing culture of *Candidatus* Arcobacter sulfidicus (60), we can calculate a specific CO_2 fixation rate of 110 nmol min^{-1} mg cell $protein^{-1}$. The minimal specific activities of the enzymes should therefore be 37 nmol min^{-1} mg cell $protein^{-1}$. The measured specific activities of all enzymes of the reductive TCA cycle in *Candidatus* Arcobacter sulfidicus were higher than this minimal activity. However, although we used purified cells of *Candidatus* Arcobacter sulfidicus for our experiments, we cannot totally exclude the growth of minor contaminants, which might have contributed to the measured enzyme activities or sequence data presented in this communication. Yet, several lines of evidence indicate that the results presented here can be attributed to *Candidatus* Arcobacter sulfidicus. First, possible contaminants constituted at most 0.1% of the total cell counts (60). Assuming we measured the enzymes of the contaminating organism(s), their enzyme activities should be at least 1,000 times higher than the measured ones, which would result in an activity of 45,000 nmol min^{-1} mg cell $protein^{-1}$ for ATP citrate lyase. To our knowledge, the highest so far measured activity of ATP citrate lyase in crude extract was 1,300 nmol min^{-1} mg cell $protein^{-1}$ (*D. hydrogenophilus*) (48). Second, based on the stable carbon isotopic composition of the total biomass formed in the reactor, we have previously determined an isotopic discrimination value of around 13 ‰ (60), which is in line with the conclusion that the dominant organism in our enrichment is using the reductive TCA cycle for autotrophic carbon fixation. A possible contaminant would not be expected to shift the stable carbon isotopic composition to that extent. Third, the obtained ATP citrate lyase sequences clearly cluster with sequences obtained from other ε-proteobacteria, which indicates that we sequenced the fragments of a ε-proteobacterium.

Another severe problem in performing physiological experiments with *Candidatus* Arcobacter sulfidicus is that for further analyses the cells have to be freed of the filamentous sulfur produced during their metabolism, resulting in a significant loss of cells and hence a low biomass available for enzyme measurements. As part of this work, we successfully established an efficient cell breakage protocol for measuring enzyme activities in samples of only 10 to 20 mg wet weight, which will also prove useful for other experiments where the amount of available biomass is an issue.

This is the first report that members of the ε subdivision of the proteobacteria use the reductive TCA cycle for autotrophic carbon fixation, and it confirms earlier results for *Candidatus* Arcobacter sulfidicus that indicated that this organism is using a carbon fixation pathway other than the Calvin cycle for autotrophic CO_2 fixation (60). It should be noted that *T. denitrificans* belongs to the ε subdivision of the proteobacteria (39), whereas the *Thiomicrospira* species belonging to the γ subdivision of the proteobacteria have been shown to use the Calvin cycle for autotrophic carbon fixation (7). Thus our data reemphasize the need to reclassify *T. denitrificans*. Based on the presence of the ATP citrate lyase gene, it was previously postulated that bacteria of the ε subdivision of the proteobacteria might use the reductive TCA cycle for autotrophic growth, but no biochemical data were presented (10). Although indicative for this pathway, the presence of this gene is not sufficient to unequivocally demonstrate that the reductive TCA cycle is used as an autotrophic pathway. For example, the obligate organotrophic bacterium *D. postgatei* uses the reductive TCA cycle for acetate metabolism (37) and the facultative autotroph *D. hydrogenophilus* has been shown to use ATP citrate lyase in both directions, depending on whether it grows autotrophically or with acetate as the carbon source (48).

Based on our data, *Candidatus* Arcobacter sulfidicus and *T. denitrificans*—like *C. limicola* and *C. tepidum*—use ATP citrate lyase for the cleavage of citrate. In contrast, *H. thermophilus*, a member of the *Aquificales*, has recently been shown to use citryl-CoA synthetase and citryl-CoA lyase for the same reaction (2, 3). It has been speculated that the reaction sequence present in *H. thermophilus* might represent an ancient form of the reductive TCA cycle and that the use of ATP citrate lyase like in *Chlorobium* was a later innovation (2, 3). This scenario is in line with 16S rRNA phylogeny, which places *H. thermophilus* at the base of the bacterial domain (40). In this regard, it is interesting to consider the phylogenetic relationship between δ- and ε-proteobacteria, which are currently the only subdivisions within the proteobacteria to have members using the reductive TCA cycle. It has been hypothesized that ε-proteobacteria, together with δ-proteobacteria, represent the most ancient subdivision of the proteobacteria and that together they might have shared a common ancestor with a group containing green sulfur bacteria and *Aquificales* (19), taxonomic groups encompassing several species which use the reductive TCA cycle for autotrophic carbon fixation. The finding of the reductive TCA cycle in ε-proteobacteria as described here is in line with this hypothesis. However, the database of prokaryotic *acl* sequences is very limited at this point and the exact reconstruction of the evolutionary history of ATP citrate lyase has to await further sequence data.

Our data lend support to the argument that autotrophic carbon fixation through the reductive TCA cycle is widespread and possibly more important in different environments than previously thought. This seems to be especially true for hydrothermal environments, where autotrophic ε-proteobacteria probably contribute substantially to primary production (10, 53). ε-Proteobacteria have been identified as a major, if not dominant, component of microbial communities in deep-sea hydrothermal vents, ranging from black smoker chimney walls and associations with invertebrates to the shallow subsurface (20, 22, 23, 41, 44). The habitat of these microorganisms seems to be confined to the zone where hydrothermal fluids mixes with ambient seawater, creating conditions that are characterized by temperatures in the range from ~20 to ~60°C and microaerobic to anaerobic conditions. Since organisms living under these conditions are potentially energy limited, it seems fitting that these microbes are using a carbon fixation pathway other than the Calvin cycle, which requires 9 ATP to synthesize one triose phosphate molecule compared to 5 ATP for the reductive TCA cycle (33).

At present, the reductive TCA cycle is the only confirmed autotrophic pathway in ε-proteobacteria and it is not known whether other autotrophic ε-proteobacteria might use a different carbon fixation pathway or not. For example, it is known that autotrophic members of the δ-proteobacteria, which represent a sister group of ε-proteobacteria, use either the reductive TCA cycle or the reductive acetyl-CoA pathway for CO_2 fixation (47, 48). Current work aims at identifying the carbon fixation pathway in other autotrophic ε-proteobacteria, including recent isolates from hydrothermal environments. This will not only result in a better understanding of the carbon fixation pathways used by ε-proteobacteria, but also result in an increased database of *acl* sequences, which is needed to reconstruct the evolution of ATP citrate lyase. However, it is interesting to note that the phylogeny based on presently available *aclAB* sequences is in congruence with 16S/18S rRNA phylogeny, indicating the potential usefulness of this gene not only as a functional but also a phylogenetic marker. The origin of ATP citrate lyase in eukaryotes also remains an unresolved and interesting question, and in this context it will be particularly helpful to obtain *acl* sequences from deep-branching microbial eukaryotes.

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